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ABNORMAL GAMETOGENESIS AND SEX RATIO IN TRIPLOID SOFT-SHELL CLAMS (*MYA ARENARIA*)*

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Abstract

Triploid soft-shell clams (*Mya arenaria*) were produced by inhibiting polar body extrusion with cytochalasin B immediately after fertilization. Diploid and triploid clams grown in suspended tray culture were examined histologically during the reproductive season of their second year. Most diploids had matured by the end of May and displayed normal gametogenic development in every individual. Triploids did not mature and most had undeveloped gonads. The few triploid females which developed some oocytes by the end of May had abnormal maturation. Triploids were 77% female as judged by the presence of oocytes; another 16% were female-like and may have been intersexes; the sex of 6% could not be identified because there was no sexual differentiation. Sex determination in this species, based on the sex ratio in triploids, best fits the model of an X:autosome balance mechanism as exemplified in some insect species.

Introduction

Mya arenaria is a deep-burrowing infaunal bivalve found ubiquitously on coastlines of the Northern Hemisphere. It is the basis of a large commercial fishery along the East Coast of North America. Recent efforts to replant clam flats led to investigations into the feasibility of producing and seeding triploid clams.

The utility of induced triploidy to aquaculture or management is founded on the supposition that triploids are sterile (Thorgaard and Allen, in press). In several species of teleosts, triploid males characteristically develop small testes and females have rudimentary ovaries (Thorgaard and Gall, 1979; Lincoln, 1981a, b; Wolters *et al.*, 1982; Yamazaki, 1983; Benfy and Sutterlin, 1984). The production of all-female triploids has thus been embraced as a guarantee of sterility (Lincoln and Scott, 1983). Sexual maturation is inhibited in both sexes by the arrest of gametogenesis in the gonial stages. The sex ratio of triploid teleosts does not depart from the 1:1 ratio expected of a homogametic female sex determining mechanism (Swarup, 1959; Gervai *et al.*, 1980; Wolters *et al.*, 1982; Yamazaki, 1983) except in the case of triploid plaice: Lincoln (1981b) reported 20 males and 1 female.

Induced triploidy in shellfish has only recently been described (Stanley *et al.*, 1981; Allen *et al.*, 1982; Chaiton and Allen, 1985) so comparisons of diploid and triploid gametogenesis are lacking. Tabarini (1984) produced triploid scallops, *Argopecten irradians*, and the majority (61% and 91% from two sampling locations) failed to ripen; 39% and 9% were reported as ripe or partially ripe. However the nature of gametogenesis

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in these functional hermaphrodites was not explored histologically. The purpose of our study was to examine the course of gametogenesis during the reproductive season in diploid and triploid soft-shell clams.

Materials and Methods

Triploidy was induced in soft-shell clam eggs by inhibiting the extrusion of a polar body with cytochalasin B (Allen *et al.*, 1982). Fertilized eggs were treated with cytochalasin either from 0 to 15 or 15 to 30 minutes after fertilization. Experimental groups and a control group—treated only with DMSO, the carrier for cytochalasin—were reared at the I.C. Darling Center, Walpole, Maine, for two years. Triploids resulting from the two treatments were sampled at four intervals during the reproductive season (3/22/82, 4/12/82, 5/4/82, and 5/25/82) and were prepared for histological examination.

Triploidy was confirmed for all experimental clams except those samples on 5/4/82. Siphons were removed and frozen for determination of ploidy by electrophoresis (Allen *et al.*, 1982) or flow cytometry (Allen, 1983).

For histological examination of gonads the body of the clam was fixed for 12 hours in Helly's Fixative (Humason, 1979), washed 12–24 hours in 3% potassium dichromate, followed by a 12 hour wash in tap water before standard dehydration and imbedding series. Slides were prepared from tissues sectioned at 6 μm and stained in Heidenhain-Mallory-Azan, as described in Humason (1979). A total of 59 controls and 64 confirmed triploids were analyzed.

Gonadal maturation was assessed by assigning a state of maturity based on the degree of gametogenesis. This scale is similar to gonadal stages used by Shaw (1965), Ropes and Stickney (1965), and Porter (1974) who described diploid gametogenesis, with the following exception: we assigned an additional stage between that of Inactive and Early Active to add further resolution to the scale during early gametogenesis. Gonad formation in the soft-shell clam commences when the animal is as small as 6 mm (Coe and Turner, 1938). Early gonad formation begins with the proliferation of gonadal primordia to form "solid, cylindrical, profusely branching alveoli filled with large, vacuolated follicle cells and widely scattered gonia on the periphery" (Coe and Turner, 1938). Our analysis did not include sampling that would follow this development but there appeared to be no differences in the gross anatomical formation of the alveoli between diploids and triploids; the index below begins at the stage of sexual differentiation of the primary gonia at the periphery of the alveoli.

Males

Inactive (Fig. 1a). Undifferentiated gonia are distributed around the periphery of alveoli and widely separated by vacuolated follicle cells which fill the alveoli. Follicle cells may have two types of inclusions. Lipoid-type nutritive inclusions, derived from the thin layer of cytoplasm which surrounds the central vacuole of the follicle cells (Coe and Turner, 1938), begin to accumulate at the time of sexual differentiation, and thus are not abundant in the inactive stage. Abnormal spermatogenesis results in a second type of inclusion characterized by multinucleated cysts. These may become pycnotic and nutritive when cytolyzed in later stages of gametogenesis. Aberrant spermatogenesis begins before the onset of normal gametogenesis (Coe and Turner, 1938) and therefore is not considered part of the active cycle.

Very early active (Fig. 1b). Follicle inclusions are most numerous when the differentiated primary spermatogonia begin to divide. The secondary spermatogonia which form are no longer widely separated by follicle cells.

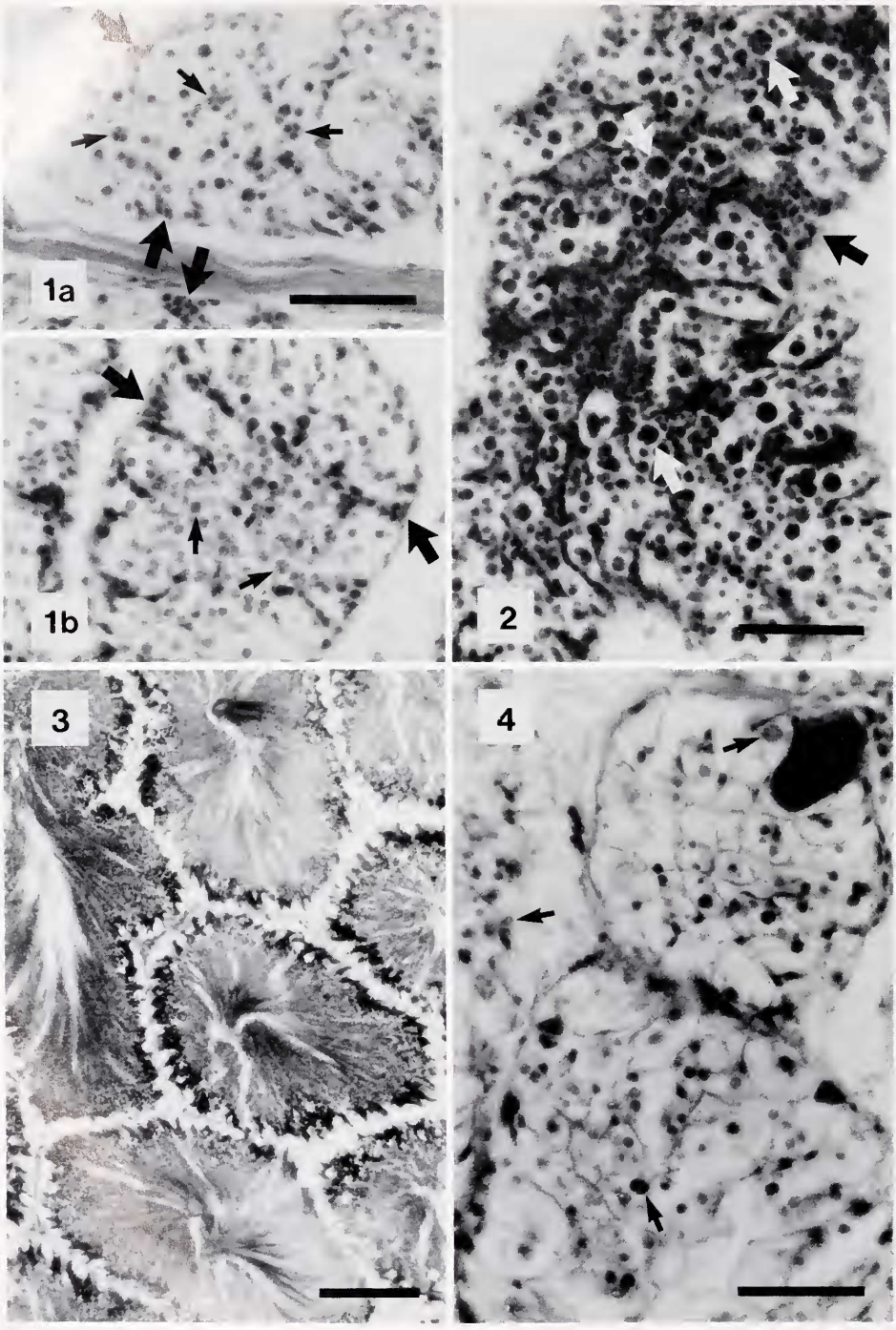

FIGURE 1a. Cross section of an alveolus in a diploid male clam in Inactive stage. Small arrows indicate multi-nucleated products of abnormal gametogenesis. Large arrows show spermatogonia. Bar = 50 μm. 250×.

Early active (Fig. 2). Primary and secondary spermatogonia proliferate in columns that invade the space occupied by the follicle cells and extend toward the developing central lumen of the alveoli. This intrusion of dividing gonia among the follicle cells characterizes mitotic divisions in the male clam. The number of successive spermatogonial mitoses may be six to nine (producing 64–512 spermatocytes), based on the range observed in other molluscan species (Roosen-Runge, 1977). Nutritive inclusions are still numerous as are products of abnormal spermatogenesis. Some spermatocytes accumulate in the center of the alveoli.

Middle active. A continuous gradient of cell types, ranging from spermatogonia at the basal membrane of the alveoli to spermatids (spermatozoa present in some alveoli) in the enlargened lumen, displaces nearly all the follicle cells. Spermatocytes and spermatids predominate. Nutritive inclusions, utilized as active gametogenesis continues, are less numerous.

Late active. Several rows of spermatogonia and/or spermatocytes remain along the basal membrane but the remainder of the alveoli are packed with radially aligned spermatids and spermatozoa. Follicle cells and inclusions are absent. The pink-stained flagella of spermatozoa extend into the central lumen of alveoli.

Ripe (Fig. 3). Alevoli are filled with radially aligned spermatozoa.

Female

Inactive (Fig. 4). As in the male, undifferentiated gonia are widely separated by follicle cells. Inclusions typical of the female include small male-type lipoid globules and larger elements, possibly albuminous, derived in part from the cytolysis of degenerating oocytes and in part from the activities of the cytoplasm of the follicle cells (Coe and Turner, 1938). Clams were identified as females by the presence of at least an occasional oocyte. We identified some indeterminate clams as probable females based on the appearance of differentiating gonia. Completely undeveloped clams could not be sexed—the primordial gonia cells and follicle inclusions in the alveoli had not proceeded to the stage of sexual differentiation.

Very early active. At least one oocyte is present in all follicles but little growth has occurred. Intact follicle cells fill alveoli.

Early active (Fig. 5). Many oocytes begin to grow (= auxocytes) and elongate toward the center of the alveoli, although lumen have not formed. All oocytes are still attached to the basal walls of alveoli. The follicle cells contain nutritive inclusions.

Middle active. Follicle cells break down, forming lumen with occasional free ova. Most of the enlarged oocytes are still attached by a slender stalk and most or all nutritive inclusions have disappeared.

FIGURE 1b. Alveolus of Very Early Active diploid male clam shows spermatogonia which have undergone several mitotic divisions (large arrows). Divisions proceeded perpendicularly to basal membrane of alveolus and invaded the space occupied by the follicle cells. High numbers of inclusions (small arrows) can be seen in follicle cells. 250×.

FIGURE 2. Early Active diploid male. Mitotic divisions are now extensive. Secondary spermatogonia extend from foci at the basal membrane of the alveolus (black arrow) into the center where they will further differentiate in later stages. White arrow indicates products of abnormal spermatogenesis that still persist. Bar = 50 μm. 250×.

FIGURE 3. Ripe diploid male. Alveoli are filled with radially aligned spermatozoa. Bar = 100 μm. 100×.

FIGURE 4. Female diploid in Inactive stage. Few oogonia are present although one growing auxocyte enables classification as female. Inclusions (arrows) are not as numerous as in the male. Bar = 50 μm. 250×.

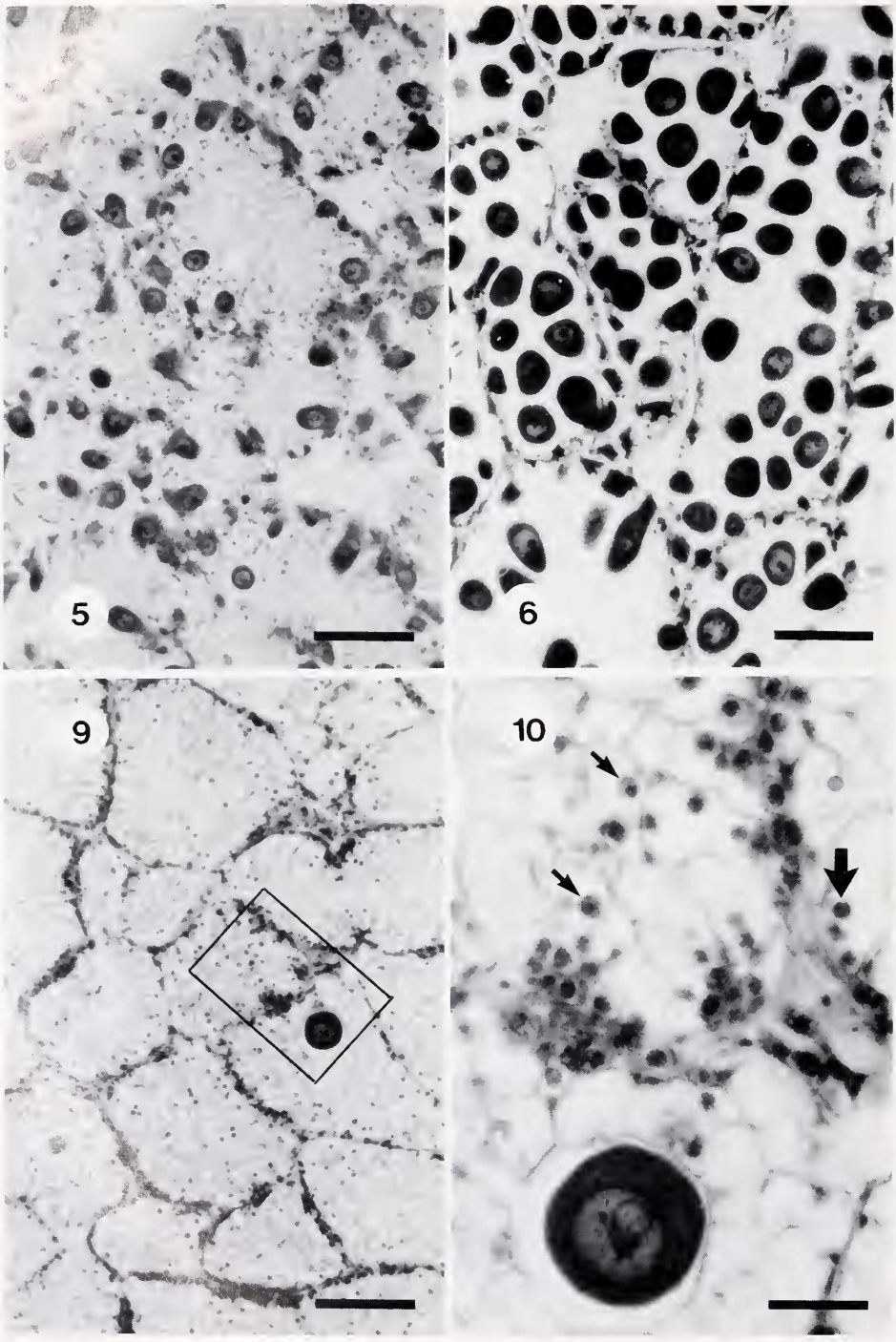

FIGURE 5. Early Active diploid female. Oogonial divisions are not normally very extensive before many developing oocytes can be seen. Bar = 100 μm. 100×.

FIGURE 6. Ripe diploid female with many free ova in alveoli. Bar = 100 μm. 100×.

Late active. Many ova have reached maximum size and are free in central lumen of alveoli. Those oocytes still attached by a slender stalk are more spherical with clearly visible nucleoli and amphinucleoli.

Ripe (Fig. 6). Most of the ova are no longer attached to the basal membrane of the alveoli. Follicle cells are absent.

Results

Proportion of triploids

When this same population of clams was sampled at 8 months (Allen *et al.*, 1982) 89.5% were triploid (n = 86) in the group treated from 0–15 minutes after fertilization and 78.5% were triploid (n = 79) in the group treated from 15–30 minutes after fertilization. In the present study 83.7% (n = 43) of those in the first treatment and 66.7% (n = 45) of those in the second treatment were triploid. A Chi square analysis comparing the proportion of triploids at 8 months with that at 2 years shows no significant differences ($\chi^2 = 1.04$, $P > .50$ and $\chi^2 = 0.81$, $P > .50$, respectively), suggesting that there is no differential mortality associated with triploidy up to first spawning.

Gametogenesis

Diploid males and females did not mature until the last sampling period when all specimens were at or past the Middle Active period (Fig. 7). In March and April only one of the 14 females and 7 of 13 males were Inactive. By May 25 the diploid population as a whole was characterized as Late Active. The triploids were largely immature. During March and April 41 of 42 triploids were classified as Inactive (Fig. 8). On May 25, 15 of 22 were still inactive. Only two triploids reached Middle Active.

None of the triploid clams were unequivocally identified as males in any of the sampling periods. Four undeveloped triploids had no sexual differentiation, as judged by the types of inclusions or by the nature of the divisions of the primary gonia. These obviously were classified as Inactive. Of the other 60 triploid clams 49 were apparently females, with definite oocytes (Figs. 9, 10). Oogonia in triploids often had a peculiar proliferation with no apparent differentiation into oocytes, resulting in pockets or clusters of oogonia at the edge of the alveoli (Fig. 10). These clusters were localized and not characteristic of the columnar intrusion into the follicle cells that is typical of male gonads.

Ten triploids did not develop oocytes but the pattern of division of the gonia, and the amount and kind of inclusions were typical of triploid females (Fig. 11). In some cases gonia proliferation would nearly fill the alveoli. Many of the cells in these clusters may have been premeiotic oocytes, *i.e.*, with large, early prophase nuclei. This feature alone however is not sufficient to discriminate between oocytes and spermatocytes. Other than that one characteristic there was no way to tell the sex of these indeterminate individuals. These 10 triploids were probably female and may have been "female" intersexes. One triploid had an atypical cytology: some of the gonia at the basal membrane of the alveoli began to divide in a manner typical of male divisions, *i.e.*, divisions

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FIGURE 9. Female triploids could be identified because of presence of occasional auxocytes which were generally nearly ripe. Edges of alveoli were heavily outlined by oogonia which presumably have divided mitotically. There were relatively few inclusions in the follicle cells. Inset of Figure 10. Bar = 100 μm. 100×.

FIGURE 10. Inset from Figure 9 at 400× shows nests of oogonia from which no oocytes are differentiating. Large arrow—possible degenerating oocyte; small arrows—non-germ cell inclusions. Bar = 25 μm.

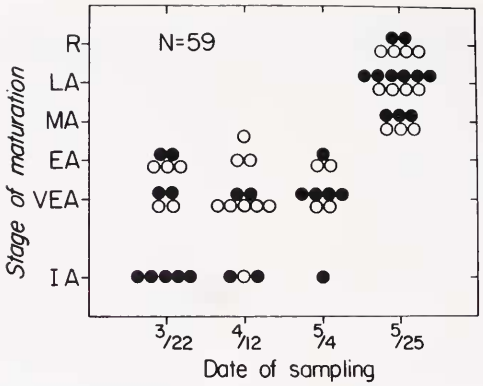

FIGURE 7. Stage of maturation (described in Materials and Methods) was plotted against sample date for males (closed circles) and females (open circles) in *diploid* clams.

progressed in columns perpendicular to the basal membrane and invaded the space occupied by follicular cells. Most of the inclusions, however, were typical of those seen in females. Furthermore non-germ cell inclusions were typical of Inactive female gonads of diploids. This individual was probably an intersexual "male."

A few females in the triploid group appear to have undergone normal or near normal early maturation (*i.e.*, five Very Early Active on 5/25). Another female (Very Early Active on 3/22) had maturing or mature eggs in the alveoli but few young oocytes. The maturation of most gonia appeared arrested. Note in Figures 12 and 13 that maturation categorized as Very Early Active and Early Active, respectively, had mature ova in the central lumen of the alveoli but few young oocytes were present. We believe that this condition was abnormal and indicative of reduced maturation. Also uncertain is whether the two Middle Active triploids observed on 5/25 (Fig. 14) were derived from further maturation of animals similar to those shown in Figures 12 and 13 or whether they represent normal maturity.

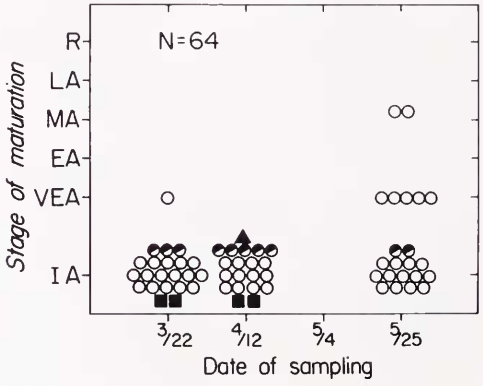

FIGURE 8. Stage of maturation was plotted against sample date for the various classes of *triploids* (described in Materials and Methods). Open circles—females; half-circles—indeterminate; square—undeveloped; and triangle—atypical cytology.

Inclusions

Follicle inclusions in the soft-shell clam serve as nutritive reserve for periods of rapid gametogenesis. Therefore the character and abundance of these substances are important to maturation. We created a scale for the abundance of inclusions based on the greatest abundance equalling 10 (found only in some males) and no inclusions—0 (as is the case in ripe or nearly ripe diploids). The values of these ratings in each sampling period were then tested non-parametrically using a two tailed Mann-Whitney test. The null hypothesis was that there was no difference in the abundance of intrafollicular inclusions between female diploids and triploids. There were significant differences between the abundance of inclusions in diploids and triploids (Fig. 15) on 3/22 ($U = 97.5$, $P < .05$), 4/12 ($U = 134.5$, $P < .01$, and 5/25 ($U = 146.5$, $P < .01$). In diploids, nutritive inclusions were accumulated at sexual differentiation and inclusions of all kinds were cytolyzed and used as gametogenesis proceeded. In triploids nutritive inclusions were relatively scarce at the beginning of the season, but because they were not utilized, they continued to accumulate. In addition products resembling those produced by abnormal gametogenesis in males also accumulated as the season progressed. By the last sampling period, triploids had significantly greater quantities of inclusions than diploids, or triploids in any other sampling period ($U = 336.5$, $P < .001$).

Discussion

Mechanisms of triploid sterility

We have demonstrated that triploid soft-shell clams, unlike their diploid cohorts, do not mature sexually late into the spawning season. Reproductive sterility is a common feature of artificially induced triploids in vertebrates. In addtion to teleosts (see Thorgaard, 1983 for review) triploid sterility has been described in Anurans (Humphrey *et al.*, 1950; Kawamura and Tokunga, 1952; Sato, 1952; and Ueda, 1980) and Urodeles, although the latter group sometimes produces functional gametes (Griffiths, 1941; Kawamura, 1940; Fankhauser and Humphrey, 1950). There are relatively few accounts of triploid sterility in invertebrates. In this report the majority of triploid *M. arenaria* had inactive gonads based on the absence of maturing gametes. Oocyte development, when it occurred, was abnormal. Only two triploids reached the stage of Middle Active.

Spermatogenesis is completely abortive in naturally occurring races of triploid trematode flatworms (Terasaki, 1977; Sakaguchi, 1980; Sakaguchi and Tada, 1980; Ramanjaneyulu and Madhavi, 1984) and cestode flatworms (Sasada, 1978; Grey and Mackiewicz, 1980). In the majority of these species meiosis ceases because homologues fail to synapse. However, in one trematode species (*Glaridacris catastomi*), multivalents form in the spermatocytes and subsequent meiotic divisions are disrupted (Grey and Mackiewicz, 1980). Meiosis in female triploid flatworms is also asynaptic and consequently embryonation begins following a mitotic maturation division. In triploid *Drosophila* females, two of the three bivalents pair at zygotene leaving the third unpaired (White, 1977). With only four pairs of chromosomes, meiotic segregation produces a relatively high proportion of functional, viable gametes. Soft-shell clams have $2N = 34$ chromosomes ($3N = 51$) (Allen *et al.*, 1982), therefore either mechanism, asynapsis or multivalent formation/aberrant segregation, would result in abnormal gametogenesis. Because we observed a few oocytes that appeared to be normal auxocytes, perhaps some gametes "develop" via the latter mechanism.

The presence of clusters of seemingly undifferentiated gonia suggests that gametogenesis in the clam is arrested, rather than uninitiated. In some cases these clusters

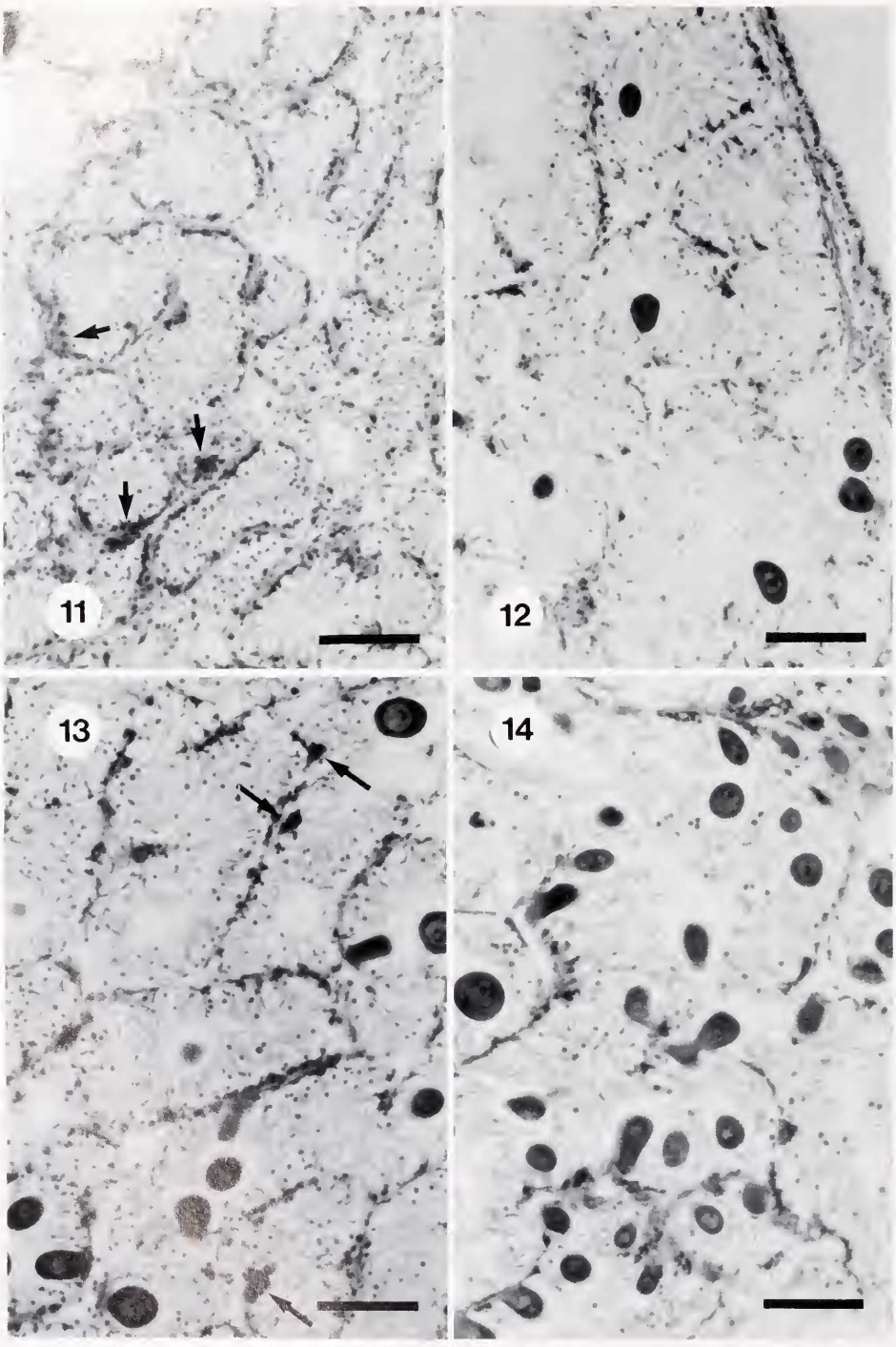

FIGURE 11. Indeterminate triploid "female" was characterized as such because of (1) the similarity in general appearance of gonads, *i.e.*, alveoli heavily outlined by gonia (nests indicated by arrows) and (2) the lack of similarity of these mitotic nests to typical male mitoses. Inclusions tended to be of non-germ cells. Bar = 100 μm. 100×.

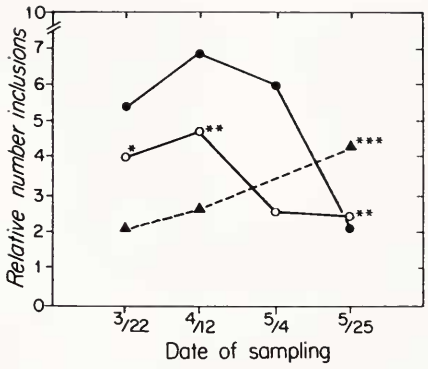

FIGURE 15. Relative number of inclusions were rated 1–10 in each specimen sampled. The means of diploid males (closed circles), diploid females (open circles), and all triploids (triangles) were plotted against sample date. Values of diploid females were tested by a Mann-Whitney test against the values of triploids, and triploids in 5/25 were tested against triploids in 4/12. *—$P < .05$; **—$P < .01$; ***—$P < .001$.

were extensive, with gonia cells nearly filling the alveolus. Lincoln (1981b) reported "nests of cells" in triploid plaice × flounder hybrids and attributed them to the persistence of secondary oogonia that had been arrested in meiosis. Gonia also proliferate in triploid *Rana pipiens* (Humphrey *et al.*, 1950) and *R. japonica* (Kawamura and Tokunga, 1952) but is followed by degeneration of premeiotic oocytes, and subsequently sex reversal (Humphrey *et al.*, 1950). Both oogonia (Raven, 1961) and spermatogonia (Roosen-Runge, 1977) undergo mitotic divisions prior to stages as oo- or spermatocytes. In diploid clams, spermatogonial proliferation was characterized by the invasion of secondary spermatogonia into the space occupied by the follicle cells; oogonial proliferation was not as extensive and was followed immediately by differentiation of premeiotic oocytes into auxocytes. Triploids identified as females (by the presence of auxocytes) shared characteristics of gross gonadal structure with triploids categorized as indeterminate (for lack of oocytes). It was this feature and not the premeiotic appearance of the gonocytes which led us to characterize indeterminates as "female" intersexes. Similarly we suggest that the clams identified as undifferentiated in early sampling periods would have "developed" into females, based on the *post facto* examination of the heavily skewed sex ratio.

Triploidy and sex ratio

We have demonstrated that at least 77% of the triploid clams are female, but 93% have histological features of females. Several correlations between the sex of triploids and sex determination can be drawn from the literature. Triploid teleosts, with one exception, have a 1:1 sex ratio confirming (Gervai *et al.*, 1980; Wolters *et al.*, 1982;

FIGURE 12. Female triploid classified as Very Early Active (VEA) although not typical of VEA in diploids. Only nearly ripe oocytes were present and (in this photo) no developing oocytes can be seen. If oocytes were not present this specimen would be classified as undeveloped. Bar = 100 μm. 100×.

FIGURE 13. Female triploid classified as Early Active because of presence of large oocytes. But as in the case of Figure 12, few young oocytes (arrows) are present. Alveoli have premature lumen. Bar = 100 μm. 100×.

FIGURE 14. Middle Active female triploid represents the most advanced stage of sexual maturity seen in the triploids sampled. Like the specimens in Figures 12 and 13 triploids displayed a tentative type of "maturity" if they began to develop at all. Figure 14 may be representative of the way some diploids mature although we saw none in our diploid samples. Bar = 100 μm. 100×.

Onozato, 1985) or suggesting (Swarup, 1959) a heterogametic male (XY) system. In triploids, XXY is male and XXX is female. In the one exception, Lincoln (1981b) could not account for an overabundance of triploid male plaice, a fish with a WZ (female heterogamety) sex-determining mechanism. The sex ratio in triploid *Rana* spp. (Anura) is variable. Triploid *R. pipiens* and *R. japonica* reverse sex from female to male prior to metamorphosis (Humphrey *et al.,* 1950; Kawamura and Tokunaga, 1952), while triploid *R. nigromaculata* show a preponderance (38:5) of males (Kawamura, 1941). However, females are most frequent in triploid *R. limnocharis* (Sato, 1952), and triploid fire bellied toads, *Bombina orientalis,* have a 1:1 sex ratio (Ueda, 1980). Males in these anurans have been shown to be the heterogametic sex (Ueda, 1980 and references therein); variability in the expression of sex ratio in triploids is apparently due to differences in the potency for sexual development mediated by the medulla of the gonad (Kawamura and Tokunga, 1952).

An alternative explanation was offered for the preponderance of females in the toad, *Bufo vulgaris formosa.* Muto (1952) suggested that the toad, like the urodeles *Triturus,* possess a WZ sex determining system. Furthermore, the second maturation division "may be reductional for W and Z," *i.e.,* following a crossover event (Muto, 1952). Suppressing this second division would yield WZ eggs, which, when fertilized by Z-bearing sperm, would produce a preponderance of females. Griffiths (1941) and Kawamura and Sanada (1949) observed mostly females in triploid *Triturus viridescens* and *T. pyrrhogaster,* respectively. However, Fischberg (1945) reported a nearly 1:1 female:male ratio in triploid *T. alpestrus.*

Sex determination in *Drosophila,* and probably in one or two branches in the phylogeny of winged insects (White, 1977), depends on the balance between the female-determining X chromosome and male-determining autosomes (A). Triploid 3X:3A individuals are all female; XXY:3A are intersexual—phenotypically intermediate between maleness and femaleness. Triploid "male" intersexes usually die but some differentiate testes which undergo abnormal spermatogenesis; XXY:3A ovaries produce mature oocytes in some cases (review by Baker and Belote, 1983). Triploid silkworms, *Bombyx mori,* develop both sexes with no intersexuality: the presence of Y (=W) in the heterogametic female is the dominant sex determining vector (Tanaka, 1953).

The genetics of sex development in the nematode, *Caenorhabditis elegans,* is particularly well studied. Normally a self-fertilizing hermaphrodite with an 2X:2A chromosome constitution, non-disjunction occasionally gives rise to X0:2A males. Triploid 3X:3A are hermaphrodites while 2X:3A individuals are male, a similar system to that of *Drosophila* (Bull, 1983). In both *C. elegans* and *Drosophila,* specific genes have been identified with major regulatory control over sexual differentiation (Baker and Belote, 1983; Doniach and Hodgkin, 1984). The products of this regulatory cascade are mediated by the "assessment" (mechanism currently unknown) of the X:A ratio.

In summation there are two major sex determining switch mechanisms (White, 1977): the dominant Y (or W, for the heterogametic female) and the genic balance system (X:A ratio). Triploids in the former system produce both males and females although sex ratios have been reported heavily skewed in one direction or the other. Triploids in the latter produce one sex and intersexes.

The soft-shell clam lends itself to a working hypothesis on sex determination because it is dioecious throughout its life, like the majority of pelecypods (Coe, 1943). The model that best accounts for our results is the X:autosome balance exemplified by *Drosophila.* The gonads of intersexes in *Drosophila* may show male or female differentiation (Baker and Belote, 1983); triploidy results in females and intersexes in an approximate 2:1 ratio because of the reduced viability of "male" intersexes. Of the 64 triploid clams about three-fourths were definite females. We expect that some

intersexual clams would appear phenotypically as females, *i.e.*, with at least one or several oocytes, leading to the overestimate of this class. We further suggest that the triploid clams labeled as indeterminate are "female" intersexes and that the atypical triploid gametogenesis seen on 4/12 was that of a "male" intersex. We found 11 of these putative intersexuals and 4 other triploids with undeveloped sex characteristics.

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